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Reconstitution of the Mitochondrial ATP-Dependent Potassium Channel into Bilayer Lipid Membrane[1]

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Electrical properties and regulation of the mitochondrial ATP-dependent potassium channel were studied. The channel protein was solubilized from the mitochondrial membrane using an ethanol/water mixture. Reconstituted into a bilayer lipid membrane BLM), the protein formed a slightly voltage-dependent channel with a conductance of 10 pS in 100 mM KCl. Often, several channels worked simultaneously (clusters) when many channels were incorporated into the BLM. The elementary channel and the clusters were both highly potassium selective. At concentrations of 1 to 10 μM, ATP favors channel opening, while channels become closed at 1–3 mM ATP. GDP (0.5 mM) reactivated the ATP-closed channels without affecting the untreated channels. The sulfhydryl-reducing agent ditiothreitol increased the open probability at concentrations of 1 to 3 mM, but damaged the selectivity of the channel.

KEY WORDS: K_{ATP}-channel; solubilization; mitochondria; bilayer lipid membrane; reconstruction.

INTRODUCTION

Two systems accomplish potassium transport in mitochondria: a uniporter provides ion influx along the electrochemical potential and a K^+/H^+ exchanger carries K^+ out of mitochondria (Rottenberg, 1973; Chaves *et al.*, 1977) Most investigations are focused now on uniporter functioning. A protein of 55 kDa was purified from mitochondria and showed uniporter properties when reconstituted into a bilayer lipid membrane (BLM) (Mironova *et al.*, 1981). To avoid contamination with detergent, which is widely used for protein solubilization but increases the nonspecific permeability of the membrane (Bangham and Lee, 1978), the protein was isolated by ethanol/water method (Mironova *et al.*, 1981,1996). Antibodies against this protein inhibited K^+ transport in mitochondria, but did not affect respiration and oxidative phosphorylation (Skarga *et al.*, 1987). When incorporated into BLM, the protein formed potassium selective channels. Channels with similar properties were observed in BLM after fusion with protein-modified liposomes (Diwan *et al.*, 1988).

The inner mitochondrial membrane is under high (200 mV) potential with minus inside. To prevent injuring accumulation of cations in mitochondria, the uniporter must be blocked by some natural inhibitor. ATP was implicated for this function. Single K^+ channel found in patches of inner mitochondrial membranes was blocked by ATP (Inoue *et al.*, 1991). ATP also inhibited the reconstituted potassium channels in liposomes (Paucek *et al.*, 1992) and BLM (Mironova *et al.* 1996).

Sensitivity to ATP indicates the mitoK channel is a member of the family of cell membrane K_{ATP} channels. This is confirmed by the inhibition of the

[1] Abbreviations used: BLM, bilayer lipid membrane; DNP, dinitrophenol; MCE, 2-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; P, coefficient of permeability; DTT, dithiothreitol.

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mitoK_{ATP} channel in mitochondria (Inoue *et al.*, 1991) and liposomes (Paucek *et al.*, 1992) by glibenclamide, a specific inhibitor of cell membrane K_{ATP} channels. However, channels in BLM formed by the 55 kDa protein that is isolated by ethanol are insensitive to glibenclamide (Mironova *et al.*, 1997). This may be caused by possible loss of the glibenclamide receptor during purification of the protein. This suggestion is supported by the observation that much higher ATP is needed for inhibition of channels formed by the 55 kDa protein in BLM as compared to inhibition of K^+ transport in proteoliposomes (Paucek *et al.*, 1992). Similarly, the cell K_{ATP} channel (Kir6.2) without glibenclamide receptor (SUR) is inhibited by ten fold higher ATP than the native KIR + SUR complex (Tucker *et al.*, 1997). It is important that conductance and selectivity of the single channel in BLM are similar to those found in intact mitochondria (Inoue *et al.*, 1991).

Regulation of the 55 kDa protein channel by nucleotides, thiol reagent, and potential is the subject of the present work. This channel is potentiated by low ATP concentration (1–10 μM) when administered to the side of membrane opposite to the side of protein addition. GDP at 0.5 mM abolished ATP inhibition but did not affect the untreated channel. Dithiothreitol, the SH-reducing agent, enhanced activity of the channel but decreased its selectivity. Similar to Kir6.2, the mitochondrial channel is weakly potential dependent.

MATERIALS AND METHODS

Mitochondria from liver of adult male Wistar rats (200–250 g) were prepared by a conventional procedure (Beavis *et al.*, 1985).

The channel protein (55kDa) was isolated by ethanol/water extraction and purified by DEAE-cellulose chromatography (Mironova *et al.*, 1996).

The ion transport activity of the protein was measured using the BLM technique (Mironova *et al.*, 1994). Lipids from the brain (20 mg/ml) and 10 % cardiolipin were dissolved in hexane and small aliquots were introduced on 1 mm aperture in the thin Teflon film separating two compartments containing 100 mM KCl in 20 mM Tris–HCl, pH 7.4. Ag–AgCl electrodes provided electrical contact between buffer and the operational amplifier (AD 711C, Analog Device, US). In the absence of the protein, the conductance of the lipid membrane was 1–3 pS. Channel protein and chemicals were added under stirring. Experiments were carried out at 20–22°C. Chemicals for electrophoresis were purchased from Bio-Rad; ATP, GTP, and DTT were from Sigma.

RESULTS

The purified 55 kDa protein is stable for 3 to 6 h at room temperature and allows measurement of channel activity during this time. Nevertheless, when stored at 4 or −20°C its activity dropped significantly during 3–4 days. There is also a problem of seasonal dependence (Fig. 1). The highest activity was observed in winter, when approximately 20–30 μg of the purified protein could be obtained from 100 mg of crude mitochondrial protein.

Protein added to one side of the BLM (*cis* side) induced mostly multichannel activity. Single channels (Fig. 2) are seen seldom. When many channels are incorporated into BLM, they switch often simultaneously (Fig. 3), assuming aggregation of several channels in a cluster. The properties of the clusters were described earlier (Mironova *et al.*, 1997a).

Current-voltage dependence displays some rectifying properties (Fig. 4) resembling that of Kir6.2 (Isomoto *et al.*, 1996). Like Kir6.2 (Tucker *et al.*, 1997), our channel is inhibited by relatively high ATP added to the trans side. The long silent channels inhibited by ATP were reactivated by GDP administered to the same compartment (Fig. 3). GDP did not affect the untreated channels (data not shown). Nucleotide

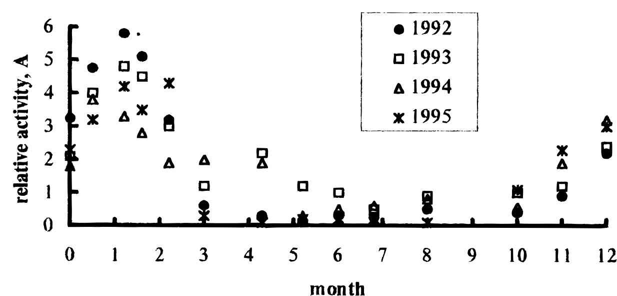


Fig. 1. Seasonal changes in the K^+ transport of protein fraction isolated from mitochondria. Seasonal changes were estimated by reconstitution of the partially purified K_{ATP} channel protein (fraction 2 after Sephadex G-15; Mironova *et al.*, 1981) into BLM. The relative activity (A) was calculated by the following formula:

$$A = V_0/V_1$$

where V_0 is the volume of the fraction 2 isolated from 400 mg of mitochondria and V_1 is the minimal volume of the fraction that increases the membrane permeability tenfold. Data of 4-year measurements are presented.

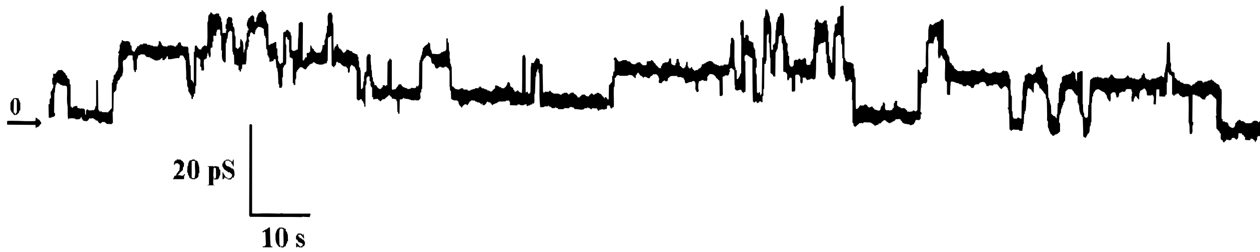


Fig. 2. Single channel activity of BLM modified with 55 kDa protein. Protein (1 μg/ml) was added to one (*cis*) side of the membrane bathed on both sides in 20 mM Tris–HCI (pH 7.4) and 100 mM KCI.

action is clearly asymmetric: the effects are seen only when nucleotides are added to the side opposite to the side of protein addition (Fig. 5). Increased ATP concentration up to 3 mM in the *cis* compartment slightly decreases channel activity when there is no ATP in the *trans* compartment. Addition of 3 mM ATP to the *trans* side inhibited channels completely.

At micromolar (1–10 μM) concentrations, ATP has potentiated the low active mitoK_{ATP} (Fig. 6). The opposite effects of low and high ATP may be essential to clarify molecular mechanisms of mitoK_{ATP} regulation. Some crucial SH-groups were found in the cytoplasmic domain of cellK_{ATP} (Islam *et al.*, 1993). Their oxidation by timerosal resulted in complete loss of channel activity, while subsequent reduction by DTT restored it. When added to mitoK_{ATP} in BLM, DTT facilitated channel opening. MitoK_{ATP} channel, as well as cytoK_{ATP} channels, often became silent (rundown) when deprived of the native cell interior. It is seen as a gradual decline of activity of the reconstituted channel. In Fig. 7, a low active mitoK_{ATP} channel, presumably because of its rundown, is activated by 1 mM DTT. This concentration of DTT did not change the background conductivity of nonmodified BLM.

Unfortunately, channel activation by DTT is accompanied by injury of channel selectivity. A twofold KCl gradient (100 mM/200 mM) caused 17 mV potential difference across the membrane with

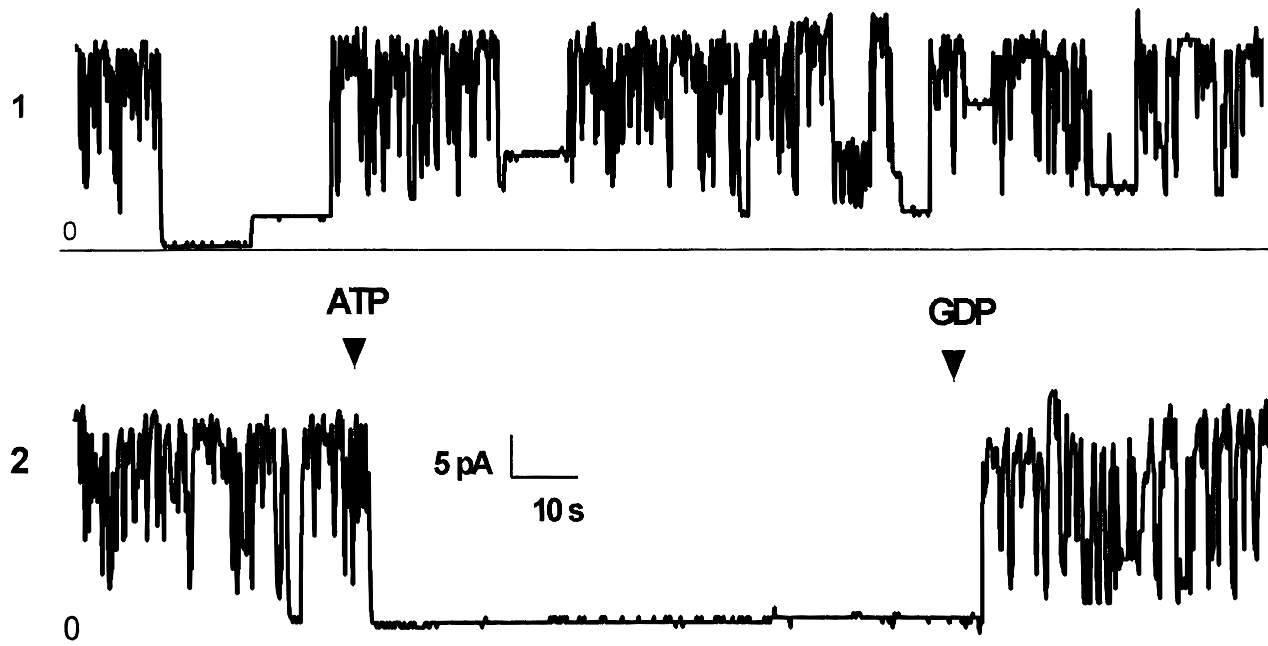


Fig. 3. Regulation by nucleotides of mitoK_{ATP} multichannel activity. (1) Clusterlike mode of several channels working simultaneously; (2) Inhibition by 2 mM ATP and reactivation by 0.5 mM GDP. Both nucleotides were added to the *trans* side at the moments indicated by arrows. Protein (3 μg/ml) was added to the *cis* compartment; buffer as in Fig. 2.

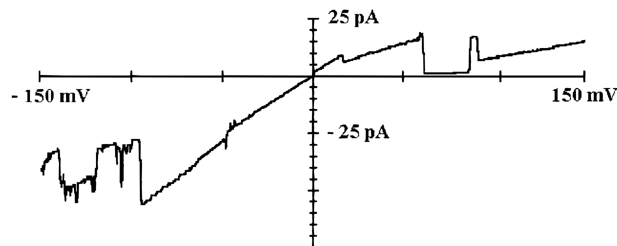
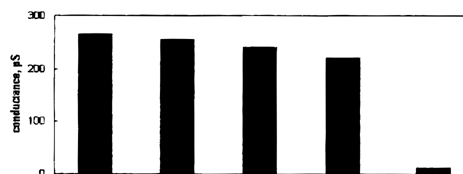


Fig. 4. Current-voltage dependence of mitoK_{ATP} channel (3 μg/ml) in symmetrical 100 mM KCl.

untreated mitoK_{ATP}. It is close to the Nernst potential. With 1 mM DTT, channel selectivity is lowered: the same KCl gradient results only in 10 mV potential, which is less than the theoretical one.

DISCUSSION

In the present work the reconstitution of the mito-K_{ATP} into BLM was perfomed. A successful study of the electrical properties and regulation of this channel was achieved due to the solubilization of the 55 kDa protein by ethanol in the absence of detergents [detergents may change the structure of isolated protein and induce a nonspecific permeability in BLM (Bangham and Lee, 1978)]. An ethanol/water mixture solubilization of some other proteins such as: Na^{+}, K^{+-} ATPase large subunit (Mironova *et al.*, 1986), Ca^{2+} uniporter from mitochondria (Mironova et al., 1994), thermogenine (Mironova *et al.*, 1992), and mitochondrial Ca^{2+}-activated channel (Mironova *et al.*, 1997b), preserved their activity in BLM. In the case of solubilization by butanol the ATPase activity of Na+,K^{+-} ATPase decreased, but in liposomes the activity was restored (Rega *et al.*, 1973).



[ATP, mM] trans side	0	0	0	0	3
[ATP, mM] cis side	0	0.5	1	3	3

Fig. 5. ATP effects depend on the side of nucleotide application. Consecutive addition of ATP up to 3 mM to the *cis* side has little effect when no ATP is in the *trans* compartment. At the *trans* side of the same membrane 3 mM ATP inhibits the channels. Conductance of the membrane with 3 μg/ml protein is presented as columns.

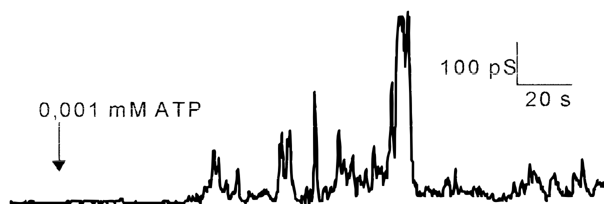


Fig. 6. Activation by low ATP of the silent mitoK_{ATP} channel. Protein (2 μg/ml) was added to the *cis* side, while ATP (1 μM) was added to the *trans* side.

Numerous data indicate that the 55kDa protein works in native mitochondria and artificial membranes as a potassium channel (uniporter). However, the functional role of this uniporter in mitochondria is not fully understood. Recently, participation of a potassium uniporter in regulation of heart activity and mitochondrial volume has been proposed (Garlid *et al.*, 1997; Garlid, 1996). An attractive idea is that futile cycling of potassium flow switches mitochondria from its role in ATP formation to that of heat production. In hibernating ground squirrels, when metabolism is slowed, the potassium transport in mitochondria is decreased as well as the channel activity in BLM of the protein fraction isolated from the same mitochondria. In mitochondria from the active animal, both of these functions are enhanced threefold. Most important, that in the intermediate state, during arousing of the animal (at animal T +28°C),when the rate of heat production is the highest, potassium transport in mitochondria and in BLM is maximal (Fedotcheva *et al.*, 1985). Seasonal variations in channel activity of the 55 kDa protein fraction (Fig. 1) also indicate the possible involvement of mitoK_{ATP} in mechanisms of heat production in mitochondria.

MitoK_{ATP} in BLM conducts potassium ions in both direction (Fig.4). It is not the result of possible loss of some regulatory part, because in the patch from one mitochondrial inner membrane, bidirectional flux

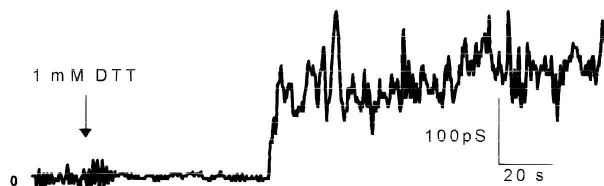


Fig. 7. Potentiating effect of dithiothreitol administered to both sides of BLM with low active mitoK_{ATP} channels. The channel was silent before DTT (1 mM) is added at the time indicated by the arrow. The other conditions are as in Fig. 2.

of K^+ has also been observed (Inoue *et al.*, 1991). Since the 55 kDa protein is under a strong electrical field in the mitochondrial membrane (negative in matrix side), it must be mainly closed in order to prevent dissipation of the electrochemical gradient. Most probably, the potassium uniporter in mitochondria is inhibited by high ATP (Inoue *et al.*, 1991; Paucek *et al.*, 1992; Mironova *et al.*, 1996), which is nearly equal on both sides of the inner mitochondrial membrane. From our data (Fig. 5), ATP is bound to one side of the protein in BLM. Whether the cytoplasmic or the matrix part of the 55 kDa protein binds ATP in mitochondria remains controversial (Inoue *et al.*, 1991; Yarov-Yarovoy *et al.*, 1997).

The rate of K^+ flux in mitochondria is low, much less than that for Ca^{2+}, but it changes in some physiological or pathological states (Howland, 1974; Zinchenko *et al*, 1982; Fedotcheva *et al.*, 1985). One of the possible candidates for the role of a natural potassium channel opener is GDP (Paucek *et al.*, 1996). In BLM, GDP abolished the ATP inhibition of mitoK_{ATP} channel (Fig. 3). Probably, some other diphosphonucleotides may act as activators as well ADP, for example, antagonized the ATP inhibition of cellK_{ATP} (Ueda *et al.*, 1997).

Molecular mechanisms of mitoK_{ATP} channel regulation are unclear. In cellK_{ATP} change of SH status modified channel activity (Islam *et al.* 1993). MitoK_{ATP} in BLM is also sensitive to SH-reducing dithiothreitol: DTT potentiates the channel (Fig. 7) but decreases its selectivity for K^+. These results correspond with the effects of small thiol-containing molecules on gating and selectivity of the bacterial potassium channel (Meury and Kepes, 1982). Some other approaches to mitoK_{ATP} channel regulation are under consideration.

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